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Abstract: **OBJECTIVES:** Macrophages (M) are one of the most important cells of the innate immune system for first line defense. Upon transplantation (Tx), M play a prominent role during lung ischaemia reperfusion (I/R) injury. Here, we hypothesize that the depletion of donor M ameliorates the post-transplant lung I/R injury. **METHODS:** Orthotopic single-lung Tx was performed between syngeneic BALB/c mice after a cold ischaemic time of 8 h and a reperfusion time of 10 h. Prior to graft implantation, alveolar macrophages of donor lungs were selectively depleted applying the 'suicide technique' by intratracheal application of clodronate liposomes (experimental, n = 6) vs the application of empty liposomes (control, n = 6). Cell count (number of F4/80(+)-macrophages) and graft injury were evaluated by histology and immunohistochemistry, and levels of lactat dehydrogenase (LDH) (apoptosis assay), enzyme linked immunosorbent assay for nuclear protein high-mobility-group-protein B1 (HMGB1), tumor necrosis factor alpha (TNF-) and transforming growth factor beta1 (TGF- 1) in plasma were analysed. **RESULTS:** Clodronate liposomes successfully reduced 70% of M from donor lungs when compared with grafts treated with empty liposome only. M-depleted transplants showed improved histology and revealed considerably less graft damage when compared with control recipients (LDH, P = 0.03; HMGB1, P = 0.3). Oxygenation capacity was ameliorated in M-depleted transplants, if not significant (P = 0.114); however, wet/dry ratio did not differ between groups (P = 0.629). The inflammatory response was significantly reduced in M-depleted mice when compared with control recipients (TNF- , P = 0.042; TGF- 1, P = 0.039). **CONCLUSIONS:** The selective depletion of M in donor lung transplants can be successfully performed and results in a sustained anti-inflammatory response upon I/R-injury. The beneficial effect of this preconditioning method should be further evaluated as a promising tool for the attenuation of I/R prior to graft implantation in clinical Tx.

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The depletion of donor macrophages reduces ischaemia-reperfusion injury after mouse lung transplantation[†]

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Abstract

OBJECTIVES: Macrophages (M) are one of the most important cells of the innate immune system for first line defense. Upon transplantation (Tx), M play a prominent role during lung ischaemia reperfusion (I/R) injury. Here, we hypothesize that the depletion of donor M ameliorates the post-transplant lung I/R injury.

METHODS: Orthotopic single-lung Tx was performed between syngeneic BALB/c mice after a cold ischaemic time of 8 h and a reperfusion time of 10 h. Prior to graft implantation, alveolar macrophages of donor lungs were selectively depleted applying the 'suicide technique' by intratracheal application of clodronate liposomes (experimental, $n = 6$) vs the application of empty liposomes (control, $n = 6$). Cell count (number of F4/80⁺-macrophages) and graft injury were evaluated by histology and immunohistochemistry, and levels of lactate dehydrogenase (LDH) (apoptosis assay), enzyme linked immunosorbent assay for nuclear protein high-mobility-group-protein B1 (HMGB1), tumor necrosis factor alpha (TNF- α) and transforming growth factor beta1 (TGF- β 1) in plasma were analysed.

RESULTS: Clodronate liposomes successfully reduced 70% of M from donor lungs when compared with grafts treated with empty liposome only. M-depleted transplants showed improved histology and revealed considerably less graft damage when compared with control recipients (LDH, $P = 0.03$; HMGB1, $P = 0.3$). Oxygenation capacity was ameliorated in M-depleted transplants, if not significant ($P = 0.114$); however, wet/dry ratio did not differ between groups ($P = 0.629$). The inflammatory response was significantly reduced in M-depleted mice when compared with control recipients (TNF- α , $P = 0.042$; TGF- β 1, $P = 0.039$).

CONCLUSIONS: The selective depletion of M in donor lung transplants can be successfully performed and results in a sustained anti-inflammatory response upon I/R-injury. The beneficial effect of this preconditioning method should be further evaluated as a promising tool for the attenuation of I/R prior to graft implantation in clinical Tx.

Keywords: Mouse • Lung • Transplantation • Macrophage depletion • Ischaemia-reperfusion injury

INTRODUCTION

Ischaemia-reperfusion (I/R) injury inevitably occurs after lung transplantation (Tx) and results in hypoxaemia, pulmonary oedema and alveolar cell damage within the first 72 h of lung Tx. This inflammatory process has been shown to show a biphasic response primarily initiated by resident macrophages (M) from the donor lung, and later be dominated by infiltrating neutrophil cells. M belong to the most important immune cells for first line defense during early I/R injury [1, 2]. These cells are a major source of a wide variety of biologically active substances, including cytokines,

chemokines but also growth factors. Once activated, they release these mediators in response to I/R-induced oxidative stress. For example, tumor necrosis factor alpha (TNF- α), released by M, has been shown to be an essential component in the cascade of inflammatory events causing lung I/R injury, not only in animal experiments [3, 4] but also in human studies [5]. When blocking TNF- α [3], using TNF- α k.o. mice [6], I/R damage could be significantly abolished. Given the presence and ability of cytokine and chemokine production by M, several M-depletion studies have been conducted with the aim of diminishing the detrimental effects of M. Indeed, it could be shown in ischaemia models that the depletion of M is protective against injury and that they are key players in the course of I/R injury development [7, 8].

However, also anti-inflammatory effects have been assigned to M. Recently, M were differentiated into various distinct functional

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subtypes with a different activation and polarization status. Classically activated M (M1) are activated by type 1 cytokines including interferon gamma and the subsequent promotion of a Th1 response. In contrast, alternatively activated M (M2) were shown to ameliorate type 1 inflammatory responses and modulate type 2 immune responses, angiogenesis and tissue repair, thereby promoting a rather protective effect to an inflammatory state.

Here, we tested in the *in vivo* model of mouse lung Tx whether the depletion of donor M has a beneficial effect on I/R injury. Unlike previously employed murine models of I/R-injury, the Tx model used here has the advantage of being able to mimic the physiological clinical condition thus making it a highly clinically relevant research model of I/R-injury in which all, ventilation and perfusion as well as cold ischaemia is applied to the engrafted organ.

MATERIALS AND METHODS

Animals, study design and mouse lung Tx model

Specific pathogen-free inbred male BALB/c mice (Harlan, Horst, Netherlands), weighing 25–32 g, were used. Animals received adequate care in strict accordance with The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, promulgated in 1985, most recently revised in 1996). The study was approved by the local veterinary ethical committee (approval number 2/2010).

Orthotopic, single-lung Tx was performed as we described [9]. Mice were orotracheally intubated with a 20-G intravenous catheter (BD Insyte, Becton Dickinson S.A., Madrid, Spain) and connected to a pressure-controlled ventilator (UNO Respirator, Amsterdam, Netherlands). Analgesia was carried out using buprenorphine 0.1 mg/kg, given 30 min prior to the thoracic surgical incision as well as 30 min before closure of the thorax and 6 h after the Tx procedure. The lungs were ventilated at a respiratory rate of 150 breaths/min and anaesthesia was maintained by ventilation with a positive end-expiratory pressure of 0.05 kPa and a tidal volume of 0.5 ml (animals weighing 25–27 g) and 1.0 ml (animals weighing 28–30 g) of 99.5% humidified, warmed oxygen with a fraction of 0.5% isoflurane (Attane; Minrad I, Buffalo, NY, USA). A heart rate of ~280 beats/min was maintained to assure stable circulation and subsequent perfusion of preserving solution throughout the pulmonary parenchyma.

The donor graft was exposed to 8 h cold ischaemia time before Tx. Grafts were analysed 3 or/and 10 h after Tx.

Macrophage depletion by clodronate liposomes

Alveolar macrophages of donor lungs were depleted by applying the 'suicide technique' through intratracheal application of liposomes. Liposomes were prepared as previously reported [10]. Liposomally encapsulated clodronate (18 mg/ml clodronate) or control empty liposome were stored at -80°C and thawed immediately prior to use. Both clodronate (1 mg/ml) and empty liposomes were prepared in 50 µl volume for intratracheal application. After intubation of donor mice, liposomes were applied via the intratracheally placed tube, followed by regular ventilation as described above. For equal distribution of the clodronate liposomes, intermittent increase in the positive end-expiratory pressure (10 times) was applied. The efficiency of clodronate liposomes was evaluated by F4/80⁺ immunohistochemistry.

Histology and immunohistochemistry for F4/80⁺ cells

Paraffin-embedded tissue sections were rehydrated and incubated with rat monoclonal anti mouse F4/80 antibody (BMA biomedical, Augst, Switzerland). We followed the manufacturer's instruction for blocking of non-specific binding and antigen retrieval (proteinase K). Primary and secondary antibody (horseradish peroxidase) bindings were visualized by 3,3'-diaminobenzidine.

Graft function

Before retrieval of the transplanted organ, mice were orotracheally intubated, connected to the pressure-controlled ventilator as described above and a laparosternotomy was performed. Graft oxygenation was evaluated by sampling blood (200 µl) directly from the left ventricle through a heparinized needle.

Wet/dry weight ratio

This assay reflects the extent of post-reperfusion pulmonary oedema. To do so, the transplanted lung was harvested, weighed and then placed in an oven at 180°C for 2 h. After the drying procedure, the transplant was weighed again, and the weight ratio before and after drying was calculated.

Tissue necrosis assay for lactat dehydrogenase

Circulating lactat dehydrogenase (LDH) was measured in heparinized mouse plasma taken from the recipient at the time of sacrifice by DRI-CHEM 400i (FUJIFILM, Tokyo, Japan).

Enzyme linked immunosorbent assay for high-mobility-group-protein B1, TNF- α and transforming growth factor beta1

Heparinized mouse plasma taken from the recipient at the time of sacrifice was used for high-mobility-group-protein B1 (HMGB1) (IBL international, Hamburg, Germany), TNF- α (Duo Set, R&D Systems, Minneapolis, MN, USA) and transforming growth factor beta1 (TGF- β 1) (Duo Set, R&D Systems) enzyme linked immunosorbent assay. Experimental procedures were followed by the manufacturer's instructions.

Statistical analysis

Data were presented as means \pm SD. Groups were compared with the Student *t*-test for unpaired samples using Prism 4.0 (GraphPad Software, San Diego, CA, USA). A two-sided *P*-value of <0.05 was considered statistically significant.

RESULTS

Table 1 summarizes the principal results obtained from macrophage-depleted (clodronate) transplants, and control transplants 10 h after reperfusion (Table 1).

Table 1: Principal results on measured parameters in macrophage-depleted (clodronate) transplants and control transplants

Parameter	Method	Results (10 h after Tx)		
		M-depleted Tx lung	Control Tx lung	P-value
M content	F4/80 ⁺ staining	30%	100%	
Lung architecture	Histology (H&E)	Less oedema Less leucocytes	Increased oedema Thickened alveolar wall	
O ₂ saturation (mmHg)	Tx oxygenation	452.7 ± 64.8	317.9 ± 69.6	0.114
Lung oedema	Wet/Dry ratio	6.87 ± 0.29	6.78 ± 0.14	0.629
LDH (plasma, U/L)	DRI-CHEM 400i	164 ± 7	439 ± 99	0.03*
HMGB1 (plasma, ng/ml)	ELISA	3.2 ± 1.2	7.2 ± 2.5	0.3
TNF-α (plasma, pg/μl)	ELISA	7.59 ± 1.79	14.45 ± 2.1	0.042*
TGF-β1 (plasma, pg/μl)	ELISA	1683.57 ± 251	3625.13 ± 458	0.039*

*Indicates significance.

M: macrophage; Tx: transplantation.

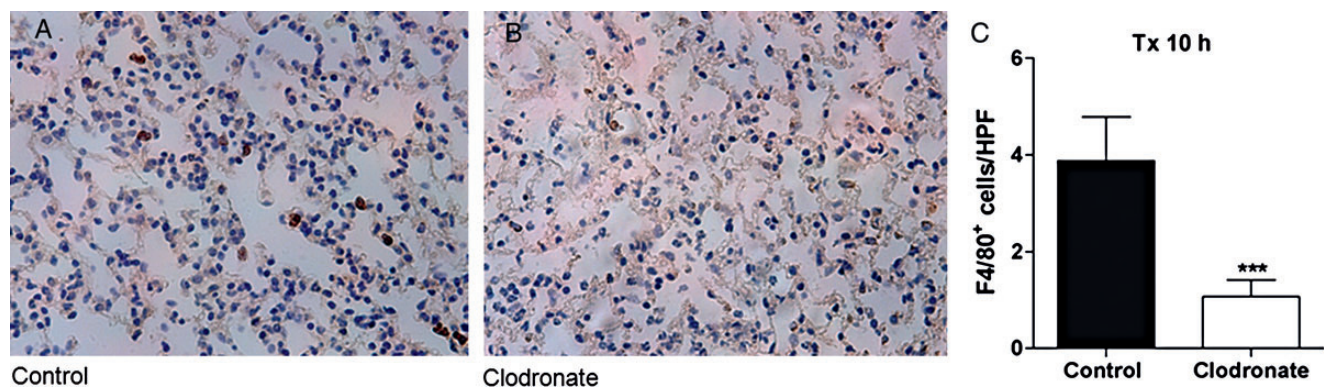


Figure 1: Clodronate liposome treatment significantly depletes alveolar macrophages from donor lungs. F4/80⁺ immune staining shows empty liposome (control: A and C). The clodronate-treated lung bears more macrophages than the control-treated lung (clodronate: B and C) 10 h after reperfusion (magnification: ×200).

Donor lung macrophages are successfully depleted by clodronate liposomes

F4/80⁺ stained macrophages were successfully depleted by clodronate liposomes at 10 h of reperfusion after a cold ischaemic time of 8 h (Fig. 1). The success rate was 70%, which corresponds to previously reported rates of M depletion [11].

Graft histology is improved upon macrophage depletion

H&E staining of lung sections from the clodronate liposome treated Tx group showed mildly less oedema, less thickening of the alveolar wall and less graft infiltrating leucocytes, when compared with the control Tx group (Fig. 2). Of note, these infiltrating cells were distinct from macrophages.

Transplant oxygenation mildly improve upon macrophage depletion

Ten hours after graft reperfusion, oxygenation slightly improved in transplanted grafts which were macrophage-depleted when compared with the control group (Fig. 3). However, this was not

significant (control: 317.9 ± 69.6 vs clodronate: 452.7 ± 64.8 mmHg, $P = 0.114$).

Graft oedema is not reduced upon macrophage depletion

The wet/dry ratios from transplanted grafts that were retrieved 10 h after reperfusion did not differ between the two groups (Fig. 4) (control: 6.78 ± 0.14 vs clodronate: 6.87 ± 0.29, $P = 0.629$).

Cell injury is reduced upon macrophage depletion

I/R injury was analysed after 8 h of cold storage and 10 h of reperfusion assessing circulating LDH and HMGB1 levels released from damaged cells. The release of the cytoplasmic enzyme LDH and the nuclear protein HMGB1 indicates an increased level of tissue damage which reflects necrosis. In line with the histological picture (Fig. 2), macrophage ablation with clodronate resulted in a significant reduction of tissue damage, which is reflected by less circulating LDH and HMGB1 levels in recipient mice (Fig. 5), (LDH control: 439 ± 99 vs clodronate: 164 ± 7 U/l, $P = 0.03$; and HMGB1 control: 7.2 ± 2.5 vs clodronate: 3.2 ± 1.2 ng/ml, $P = 0.3$ respectively).

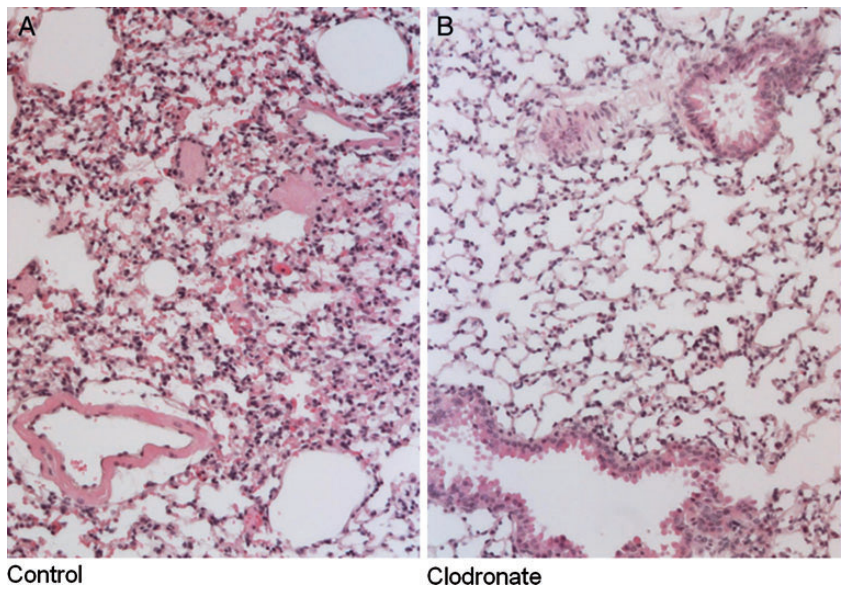


Figure 2: Graft histology is improved upon macrophage depletion. Ten hours after Tx, the reperfusion resulted in mild oedema, thickening of the alveolar wall and leucocyte influx into the parenchyma in empty liposome-treated mouse lung (control: **A**). In comparison, only slight changes were present in the clodronate-treated lung (**B**) (H&E staining, magnification: $\times 200$).

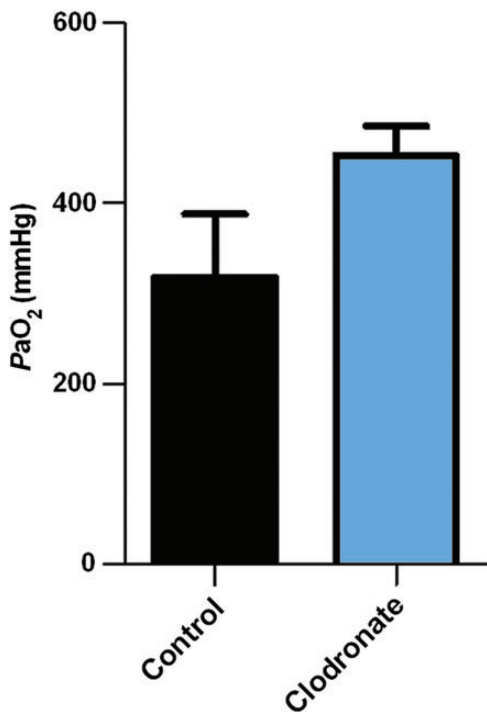


Figure 3: Graft oxygenation in control transplants vs macrophage-depleted transplants (clodronate), 10 h after reperfusion (control: 317.9 ± 69.6 vs clodronate: 452.7 ± 64.8 mmHg, $P = 0.114$). Values are expressed as mean \pm SD.

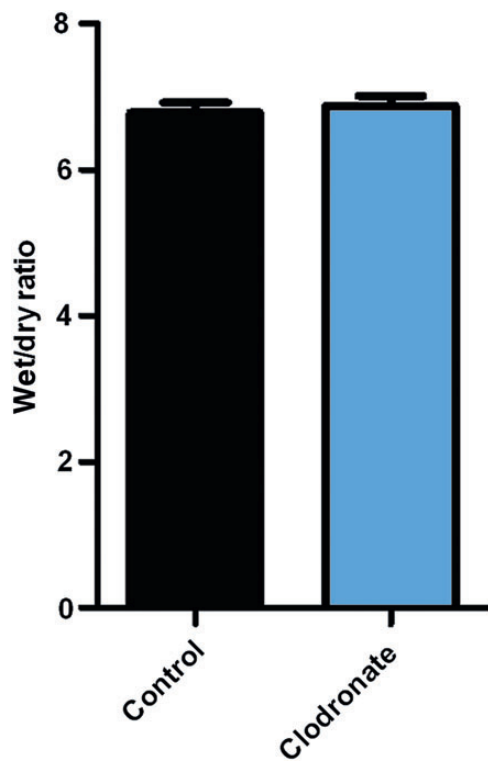


Figure 4: Wet/dry ratio in control transplants vs macrophage-depleted transplants (clodronate), 10 h after reperfusion (control: 6.78 ± 0.14 vs clodronate: 6.87 ± 0.29 , $P = 0.629$). Values are expressed as mean \pm SD.

TNF- α is reduced by macrophage depletion at late reperfusion

Proinflammatory cytokine production is a key mechanism of tissue damage after ischaemic insult. We were analysing the kinetic expression of the proinflammatory cytokine TNF- α in the plasma of recipients at 3 and 10 h upon reperfusion. The levels of circulating TNF- α

were increasing by 10 h of reperfusion in the control group. In contrast, clodronate treatment group showed declining levels of TNF- α in the circulation at 10 h of reperfusion (Fig. 6), (TNF- α : control 14.45 ± 2.1 vs clodronate 7.59 ± 1.79 pg/ μ l, $P = 0.042$). These data are consistent with our data gained in LDH-associated organ injury.

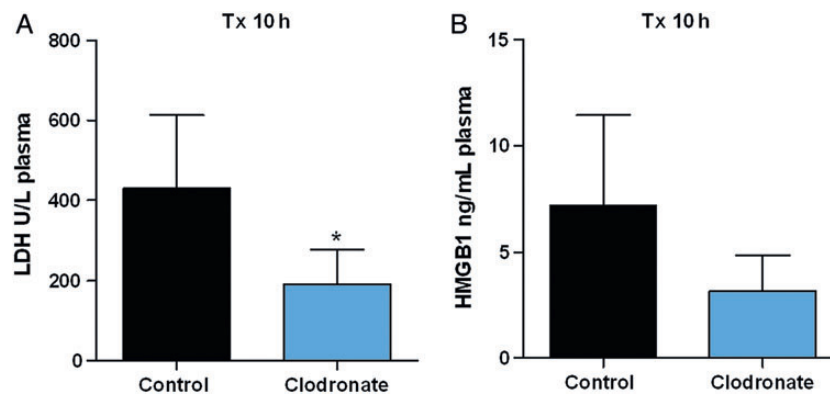


Figure 5: Cell injury is reduced upon macrophage depletion. Organ injury after 10 h of Tx was assessed by circulating LDH and HMGB1 that is released from damaged cells. Macrophage ablation with clodronate resulted in a significant reduction of circulating LDH and a non-significant reduction in HMGB1 levels in recipient mice ($P = 0.03$ and $P = 0.3$, respectively).

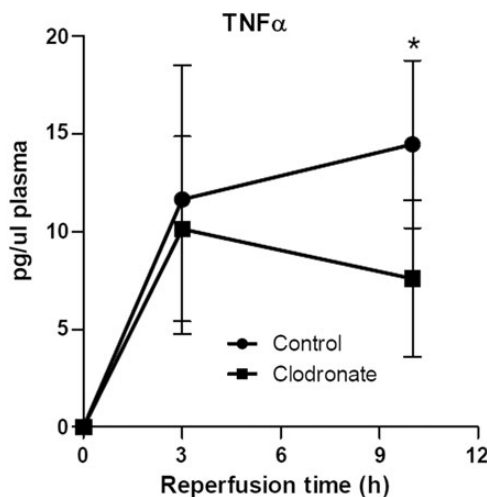


Figure 6: TNF- α level is reduced upon macrophage depletion during late reperfusion. The kinetic expression of the pro-inflammatory cytokine TNF- α was measured in the plasma of recipients at two reperfusion time points (at 3 and 10 h). Recipients receiving an M-depleted organ show a significantly reduced level of circulating TNF- α 10 h after reperfusion.

TGF- β 1 is reduced by macrophage depletion at late reperfusion

Besides its role in immune homeostasis, TGF- β 1 is recognized as a mediator of acute and also chronic lung injury through enhancement of alveolar epithelial permeability and the stimulation of the extra cellular matrix. In line with the increase in LDH and TNF- α , TGF- β 1 levels increased in the circulation at 3 h of reperfusion and (Fig. 7) were associated with mild oedema as shown by histology (Fig. 2). However, macrophage ablation by clodronate mitigated levels of circulating TGF- β 1 10 h after reperfusion (Fig. 7), (TGF- β 1 control: 3625.13 ± 458 vs clodronate: 1683.57 ± 251 pg/ μ l, $P = 0.039$).

DISCUSSION

Our study indicates that depletion of macrophages from donor lung transplants beneficially modulates the inflammatory response from I/R injury in two ways: (i) cell injury was significantly reduced as shown by less release of LDH and HMGB1 in the circulation and (ii) TNF- α and TGF- β 1 were both down-regulated in

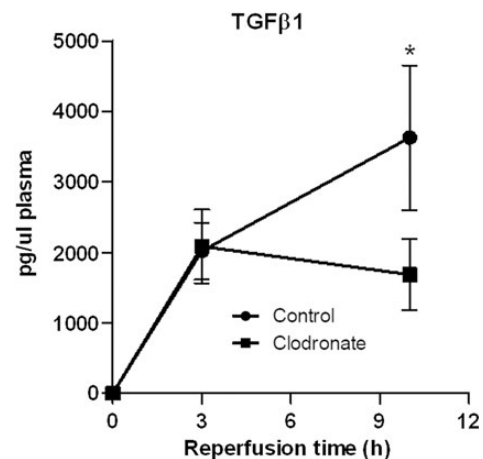


Figure 7: TGF- β 1 level is reduced by donor macrophage depletion during late reperfusion. Circulating TGF- β 1 levels increased after 3 h of reperfusion but decreased after 10 h of reperfusion, indicating a reduced inflammatory response upon Tx in M-depleted recipients

plasma at 10 h after reperfusion, reflecting a sustained suppression of inflammation in the recipient. In spite of only moderate functional improvement of the transplanted graft itself, our data suggest that donor macrophages contribute to systemic inflammation and can deteriorate graft outcome after lung I/R.

The presence of macrophages has been described to be of differential outcome in lung Tx. On the one hand, alveolar macrophages have been implicated as key initiators of acute lung injury, showing that M depletion protects the lungs from I/R-induced dysfunction and significantly reduces cytokine as well as chemokine production. Furthermore, the presence of M indicated that protein expression of TNF- α and MCP-1 are positively correlated with I/R-induced lung injury, and M are a major producer of TNF- α , MCP-1 and macrophage inflammatory proteins (MIP)-2 [8]. These data were corroborated by Naidu *et al.* [7] showing that alveolar macrophages are the key early source of multiple proinflammatory mediators and that depleting alveolar macrophages is protective against injury. In contrast, Nakamura *et al.* [12] found opposite results showing that the absence of macrophages rather aggravated I/R injury of the lung as reflected by increasing MIP-2-associated alveolar neutrophil recruitment and airway mechanical dysfunction. The models of I/R injury that were used in these studies differed from each other such as arterial perfusion occlusion only, or clamping the entire hilum thus also occluding

the ventilation. This might be responsible for the heterogeneous results reported. The advantage of our model of orthotopic single lung Tx is that all conditions equal the human Tx setting: ventilation as well as perfusion is stopped for a certain period of time and cold ischaemia time can be provided.

Besides the transcription factor NF- κ B, TNF- α is the key cytokine in the early phase of I/R-injury [13, 14], which is also in line with the data from Zhao *et al.* [8] and Naidu *et al.* [7]. Accordingly, we could show that circulatory levels of TNF- α (10 h after reperfusion) from M-depleted animals were significantly decreased upon reperfusion. In contrast, levels of TNF- α early after reperfusion (3 h) did not show any differences to controls, suggesting that other TNF- α -producing cells may play a role in the early phase. However, at a later stage, macrophages seem to be decisive in the production of this strong proinflammatory cytokine. Much to our surprise, we could not find any difference in intragraft levels of TNF- α (data not shown). Unlike systemically increase levels of TNF- α , we did not find TNF- α to be change within the transplanted graft. This result might be due to lysed macrophages-derived shedding of antigenic material which induce a systemic inflammatory response in the periphery. The depletion of M not only diminished proinflammatory cytokines and chemokines, it also has an influence on the recruitment of neutrophils, the latter being responsible for the late-phase injury [15]. Accordingly, our histology suggested an improvement of organ architecture, showing less cell infiltrates, including neutrophils. Ross *et al.* [15] showed in a rabbit model of vessel ligation that the administration of anti-TNF- α and anti-IL-1 β antibodies before reperfusion blocked neutrophil chemotaxis and chemokine and cytokine production and was responsible for less I/R-injury. Antagonizing the effects of TNF- α with an antibody was also convincingly shown by Khimenko *et al.* [16] in an *ex vivo*-perfused rat lung model of I/R-injury and by Krishnadasan *et al.* [17] in an *in vivo* model of hilum clamping.

Apoptosis is the main mechanism of cell death that occurs in I/R-injury in both experimental and human lung Tx [13]. In contrast to necrosis which occurs in ischaemia, apoptosis is present only after reperfusion and increases rapidly with a peak around 2 h after reperfusion [18]. In this context, the duration of cold ischaemia closely correlates with the proportion of apoptosis and necrosis. A cold ischaemic time up to 12 h is associated rather with an apoptotic appearance of the graft [19]. In our study, we employed 8 h of ischaemia in order to induce a marked but not disastrous injury to the graft. Yet, we could show severe cell damage by significant elevated levels of LDH released into the circulation at 10 h after reperfusion. Even worse, enhanced release of HMGB1 (a nuclear factor, released upon necrosis [20]) 10 h after reperfusion indicated that necrosis rather than apoptosis was induced during a later time point of reperfusion. Given the fact that necrosis is associated with significant worsened lung function due to high degree of inflammation [21], the reduction in HMGB1 levels due to the absence of M indicates a rather cell-protective effect.

We were not able to show an effect towards alternatively activated M of the population resting within the engrafted organ (30% of non-depleted M). TGF- β 1 is known to drive M2 differentiation from monocytes and can thereby evolve anti-inflammatory effects. But TGF- β 1 also mediates acute and chronic lung injury including alveolar flooding, increment of endothelial permeability, oedema and pulmonary fibrosis [22]. After 10 h of reperfusion, levels of circulating TGF- β 1 were reduced in the clodronate-treated group, while they were increased in the control group. We concluded from these data that the majority of the depleted

donor macrophages rather belong to the classical M population as TNF- α levels strikingly declined upon M depletion.

The M depletion method in transplant donors has the potential for major clinical impact. Fiser *et al.* [23] successfully depleted donor M by using gadolinium chloride (GDC) and showed that reperfusion injury was attenuated at an early time point. However, the potential application of clodronate is more likely to be used in the Tx setting, as GDC must be given 24 h in advance to effectively limit macrophage functionality. In contrast, clodronate induces macrophage lysis within 6 h of application. Given the time frame from organ retrieval to implant into the recipient of a maximum of 6 h, it is well imaginable that clodronate can be administered upon organ harvesting in order to develop its efficacy during this period.

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Conflict of interest: None declared.

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